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Expression of Interleukin-8 by Human Melanoma Cells Up-Regulates MMP-2 Activity and Increases Tumor Growth and Metastasis

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Expression of interleukin-8 (IL-8) by human melanoma cells correlates with their metastatic potential. Moreover, UV-B irradiation of primary cutaneous melanoma cells induces IL-8 mRNA and protein production and increases both tumor growth and metastasis in nude mice. Although IL-8 has been shown to be an angiogenic factor, the biological consequences of increased IL-8 production by melanoma cells and the role of IL-8 in the metastatic process remains unclear. The purpose of this study was to determine the role of IL-8 in tumor growth and metastasis of human melanoma cells. Nonmetastatic SB-2 melanoma cells with negligible levels of IL-8 were transfected with IL-8 cDNA and subsequently analyzed for changes in their tumorigenic and metastatic potential. Enforced expression of IL-8 rendered the melanoma cells highly tumorigenic and increased their metastatic potential as compared with parental and control transfected cells. The IL-8-transfected cells displayed up-regulation in M_r 72,000 collagenase type IV (MMP-2) mRNA and collagenase activity and increased invasiveness through Matrigel-coated filters. Moreover, when the MMP-2 promoter was linked upstream of the chloramphenicol acetyltransferase (CAT) reporter gene, CAT activity was up-regulated in IL-8 but not in control transfected cells, suggesting that IL-8 is involved in MMP-2 gene transcription. Activation of type IV collagenase by IL-8 can enhance the invasion of host stroma by the tumor cells and increase angiogenesis and, hence, metastasis. *(Am J Pathol 1997, 151:1105–1113)*

The production of metastases depends on the completion of a multistep process involving the survival and growth of unique subpopulations of cells with metastatic properties.[1] The growth and progression of tumor cells require autocrine growth factor production or the ability to respond to external stimuli such as hormones or growth and differentiation factors.[2]

Melanoma cells secrete a variety of growth factors either constitutively or subsequent to induction by other cytokines.[3–5] Among the cytokines secreted by melanoma cells are transforming growth factor-α,[6] transforming growth factor-β,[7] platelet-derived growth factors A and B,[8] basic fibroblast growth factor (bFGF),[9] interleukin (IL)-1,[10,11] IL-6,[4,12] IL-10,[13,14] granulocyte/macrophage colony-stimulating factor,[15] and melanoma growth-stimulating activity (MGSA).[6] These growth factors/cytokines may act as autocrine growth factors or act in paracrine fashion on the host environment to stimulate growth.[5] Increased proliferation, however, is not enough to give rise to metastases. To possess metastatic potential, a cell has to be able to invade the surrounding tissue, spread via lymphatics and/or the bloodstream, extravasate, and multiply at a secondary site. Genes involved in cell attachment, motility, and proteolytic degradation of the extracellular matrix are likely to be important in these processes.

IL-8 was originally identified as a leukocyte chemoattractant,[16] and it shares 44% amino acid homology with MGSA/gro, an autocrine growth factor for melanoma cells.[17] IL-8 has been shown to induce both angiogenesis[18,19] and haptotactic migration in melanoma cells.[20] As angiogenesis, migration, and cell proliferation are all important components of the metastatic process, IL-8 expression by melanoma cells could influence their metastatic capabilities. Indeed, it has been shown that the constitutive expression of IL-8 in human melanoma cells directly correlates with their metastatic potential in nude mice.[21] In addition, we recently demonstrated that UV-B irradiation of primary cutaneous melanoma cells rendered them highly tumorigenic and increased their metastatic potential via the induction of IL-8.[22] These data provide indirect evidence that the development of metastatic capacity in melanoma cells may be associated with an increase in IL-8 expression, but whether these observations are causally related to tumor growth and metas-

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tasis of human melanoma and the exact mechanism by which IL-8 exerts its angiogenic effect remain unclear.

In the present report, we provide direct evidence that expression of IL-8 in low-tumorigenic nonmetastatic SB-2 cutaneous melanoma cells (with negligible levels of IL-8) significantly increased their tumorigenicity and metastatic potential in nude mice. The transfected cells exhibited increased activity and mRNA production of the M_r 72,000 collagenase type IV (MMP-2). Moreover, chloramphenicol acetyltransferase (CAT) activity driven by the MMP-2 promoter was up-regulated in IL-8-transfected cells and in parental SB-2 cells treated with exogenous recombinant human IL-8. Our data suggest a potential mechanism by which IL-8 exerts its angiogenic effect and the increased metastatic potential of tumor cells expressing this cytokine.

Materials and Methods

Cell Line and Culture Conditions

The SB-2 cell line was isolated from a primary cutaneous lesion[23] and was a gift from Dr. B. Giovanella (St. Joseph's Hospital Cancer Center, Houston, TX). The cells were maintained in culture as adherent monolayers in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, twofold vitamin solution, and penicillin-streptomycin (Flow Laboratories, Rockville, MD). All cultures were free of *Mycoplasma* and pathogenic murine viruses (assayed by Microbiological Associates, Bethesda, MD). Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

Animals

Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific-pathogen-free conditions and used at 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Expression Vectors and Cell Transfections

A full-length IL-8 cDNA (*Eco*RI-*Eco*RI, 1.5 kb, a gift of Dr. K. Matsushima, Kanazawa, Japan[16]) was inserted into the *Eco*RI site of pUC19. A *Sma*I-*Bam*HI fragment (~0.8 kb) was cloned into the *Sma*I-*Bam*HI sites of Bluescript KS(+) (Stratagene, La Jolla, CA). The resultant Bluescript KS(+) was digested with *Xho*I-*Not*I and cloned into the *Xho*I-*Not*I site of the BCMGS-Neo expression vector (Stratagene). Expression of IL-8 cDNA was under the control of the cytomegalovirus promoter. SB-2 melanoma cells were transfected by using the Lipofectin reagent (GIBCO-BRL, Gaithersburg, MD) and 5 μg of DNA. Transfections were carried out as we described previously.[26] After 48 hours, fresh medium containing 600 μg/ml active G418 was added; cultures were maintained in this medium. Fourteen days later, neo-resistant colonies were isolated by trypsinization and established in culture.

Northern Blot Analysis

mRNA was isolated by FastTrack 2.0 (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Three micrograms of mRNA/lane was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 amp to NYTRAN nylon transfer membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked with 120,000 μJ/cm^2 by using a UV Stratalinker 1800 (Stratagene). Hybridizations were performed as described previously.[22] Nylon filters were washed three times at 55 to 60°C with 30 mmol/L NaCl/3 mmol/L sodium citrate (pH 7.2)/0.1% SDS (w/v). The cDNA probes used in these analyses were a 1.3-kb *Pst*I cDNA fragment corresponding to rat glyceraldehyde 3-phosphate dehydrogenase[24] and a 0.5-kb *Eco*RI cDNA fragment corresponding to human IL-8.[16] Each cDNA fragment was purified by agarose gel electrophoresis, recovered using Qiaquick (QIAGEN, Chatsworth, CA), and radiolabeled using the random primer technique with [α^{32}P]deoxyribonucleotide triphosphates.

IL-8 mRNA expression was quantified in the linear range of the film on a Personal Densitometer using the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA). Each sample measurement was calculated as the ratio of area between the 1.8-kb IL-8-specific mRNA transcript and the 1.3-kb glyceraldehyde 3-phosphate dehydrogenase transcript.

Enzyme-Linked Immunosorbent Assay (ELISA) for Human IL-8

IL-8 levels in cell culture supernatants from different treatments were determined by using an ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). This assay is a quantitative immunometric sandwich enzyme immunoassay. A curve of the absorbance *versus* the concentration of IL-8 in the standard wells was plotted. By comparing the absorbance of the samples to the standard curve, we determined the concentration of IL-8 in the unknown samples.

Chemotaxis Assay for IL-8

Supernatants from melanoma cells (1×10^6) grown in minimal essential medium supplemented with 10% fetal bovine serum were collected after 24 hours and placed in the lower compartment of the Boyden chambers and served as a source of chemoattractants. Human polymorphonuclear cells (PMNs; 1.5×10^5) isolated from a healthy donor by the dextran gradient method were suspended in Hanks' balanced salt solution (HBSS) and

placed on top of polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size; Nucleopore, Pleasanton, CA). After incubation for 1 hour at 37°C, the cells on the lower surface of the filters were stained with Diff-Quick and quantified with an image analyzer (Optomax V) attached to an Olympus CK2 microscope. The data were expressed as the average number of cells on the lower surface of the filter from each of triplicate experiments ± SEM.

Tumor Cell Injections

To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). The flask was sharply tapped to dislodge the cells, and supplemented medium was added. The cell suspension was pipetted to produce a single-cell suspension. The cells were washed and resuspended in Ca^{2+}/Mg^{2+}-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used. Subcutaneous (s.c.) tumors were produced by injecting 1×10^6 tumor cells/0.2 ml HBSS over the right scapular region. Growth of s.c. tumors was monitored by weekly examination of the mice and measurement of tumors with calipers. The mice were killed 2 months after injection, and tumors were processed for hematoxylin and eosin (H&E) staining.

For experimental lung metastasis, 1×10^6 tumor cells/0.2 ml of HBSS were injected into the lateral tail vein of nude mice. The mice were killed after 60 days, and the lungs were removed, washed in water, and fixed with Bouin's solution for 24 hours to facilitate counting of tumor nodules as we described previously.[25,26] The number of surface tumor nodules was counted under a dissecting microscope. Sections of the lungs were stained with H&E to confirm that the nodules were melanoma and to monitor the presence of micrometastasis.

Zymography

Collagenolytic activity was determined on substrate-impregnated gels,[27] with minor modifications. A total of 5×10^3 cells/well were replated into a 96-well culture plate. After 24 hours of cell adherence, cultures were washed and re-fed with fresh medium. Culture supernatants were collected and analyzed for collagenase activity. Samples of culture media were separated on gelatin-impregnated (1 mg/ml; Difco, Detroit, MI) SDS/8% polyacrylamide gels under nonreducing conditions, followed by 30 minutes of shaking in 2.5% Triton X-100 (BDH, Poole, UK). The gels were then incubated for 16 hours at 37°C in 50 mmol/L Tris, 0.2 mol/L NaCl, 5 mmol/L $CaCl_2$, and 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H_2O (30:10:60). The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A).

DNA Transfection and CAT Assays

Using the lipofectin protocol (GIBCO BRL), we transfected the basic CAT expression vector with no promoter/enhancer sequences (pCAT-basic) or a control plasmid with SV40 promoter and enhancer (pCAT-control; Promega, Madison, WI) into SB-2 parental neo-transfected and IL-8-transfected SB-2 IL-8 H5 cells. One copy of the human MMP-2 promoter region[28] spanning nucleotides −390 to +290 was ligated upstream of the basic CAT expression vector. The human MMP-2 promoter segment was generated by the polymerase chain reaction using primers encompassing both ends of this domain and sticky ends of *Hin*dIII and *Xba*I, respectively, as we previously described for the *p53* promoter.[29] We transfected 3.0×10^6 cells/well of a six-well tissue culture dish with 2.5 μg of the reporter CAT constructs and 2.5 μg of a β-galactosidase (β-gal) expression plasmid. The β-gal expression vector is controlled by the viral thymidine kinase promoter (Clontech, Palo Alto, CA). After 48 hours, extracts were prepared from all plates, normalized for β-gal activity, and assayed for CAT activity[30] as we described previously.[29] Each assay was repeated at least three times; there was less than 10% variation among individual transfections. The CAT assay was quantified by densitometry (Personal Densitometer, Molecular Dynamics).

Invasion Assay through Matrigel

Polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size; Nucleopore) were coated with a mixture of basement membrane components (Matrigel, 25 μg/filter) and placed in modified Boyden chambers. The cells (2×10^5) were released from their culture dishes by short exposure to EDTA (1 mmol/L), centrifuged, resuspended in 0.1% bovine serum albumin/Dulbecco's minimal essential medium, and placed in the upper compartment of the Boyden chamber. Fibroblast-conditioned medium was placed in the lower compartment as a source of chemoattractants. After incubation for 6 hours at 37°C, the cells on the lower surface of the filter were stained with Diff-Quick (American Scientific Products, McGaw Park, IL) and quantified with an image analyzer (Optomax V) attached to an Olympus CK2 microscope. The data were expressed as the average number of cells on the lower surface of the filter from each of the three experiments ± SEM.

Results

Expression of IL-8 in SB-2 Human Melanoma Cells

To assess the effect of IL-8 expression on tumor growth and metastasis of human melanoma cells, we chose to work with the SB-2 cell line. SB-2 cells originated from primary cutaneous melanoma[23] and are low-tumorigenic and nonmetastatic in nude mice.[22,31,32] In addition, SB-2 is the only cell line that exhibits negligible levels of IL-8

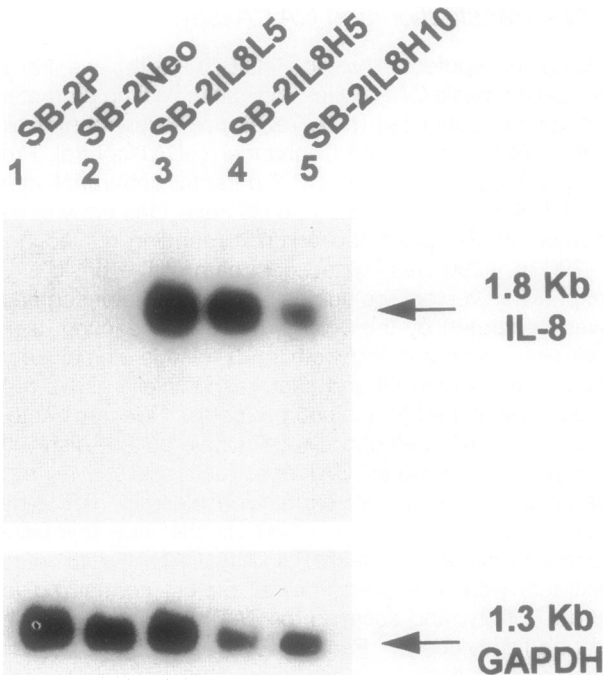

Figure 1. Northern blot analysis for the expression of IL-8 in parental SB-2 cells (**lane 1**), in neo-transfected cells (**lane 2**), and in three IL-8-transfected clones (**lanes 3** to **5**). The same blot was hybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe to verify the integrity of RNA and the amount loaded in each lane.

mRNA expression and protein secretion.[22] The other 12 human melanoma cell lines that originated from metastatic lesions produced and secreted IL-8, and the level of IL-8 expression correlates with their metastatic potential *in vivo*.[21] After gene transfection with the cDNA encoding human IL-8, three neo-resistant clones transfected with IL-8 and one control clone transfected with the naked expression vector alone (neo-control, without IL-8 cDNA) were analyzed for IL-8 expression. Northern blot analysis using the IL-8 cDNA as a probe detected appreciable levels of mRNA transcripts in all three clones, designated as SB-2 IL-8-L5, SB-2 IL-8-H5, and SB-2 IL-8-H10 (Figure 1, lanes 3 to 5, respectively), but not in wild-type SB-2 cells or in cells transfected with expression vector alone (Figure 1, lanes 1 and 2, respectively). Secretion of IL-8 by the transfected clones was assayed by ELISA. The three isolated clones were found to secrete IL-8 in the range of 833 to 1356 pg/ml per 10^6 cells every 24 hours as compared with 36 to 39 pg/ml/10^6/24 hours secreted by the parental and neo-transfected cells (Table 1). To demonstrate that the IL-8 secreted by the transfected cells is biologically active, we analyzed its ability to act as a chemotactic factor for human PMNs. Table 2 shows that supernatants from the three IL-8-transfected clones possess chemotactic activity for human PMNs as compared with supernatants collected from parental SB-2 and neo-transfected cells ($P < 0.001$).

Table 1. Secretion of IL-8 Protein by SB-2 Cells Transfected with the IL-8 Gene

Cell lines	Secretion of IL-8 protein (pg/ml) per 10^6 cells/24 hours
SB-2 parental	36.5 ± 3.0
SB-2 neo	39.7 ± 1.0
SB-2 IL-8-H5	1,120 ± 10.0
SB-2 IL-8-L5	1,356 ± 13.0
SB-2 IL-8-H10	833.0 ± 9.0

A total of 1×10^6 cells/well were cultured. After 24 hours of cell adherence, cultures were washed and re-fed with fresh medium. Culture supernatants were collected and analyzed for IL-8 secretion by ELISA. Values are mean ± SD of duplicate cultures.

Tumorigenicity of IL-8-Transfected Cells

To determine the tumorigenicity of the IL-8-transfected clones, we injected 1×10^6 cells from the SB-2 IL-8-H5 and SB-2 IL-8-L5 clones s.c. into BALB/c nude mice. Tumor growth was monitored once a week (Figure 2), and the tumors were harvested to analyze IL-8 expression *in vivo* once they reached 0.6 cm in mean diameter. The results summarized in Figure 2 show that parental and neo-transfected control cells did not begin to form palpable tumors until 56 days after injection; these results confirmed our previously published data.[22,31,32] In contrast, both IL-8-transfected clones, SB-2 IL-8-H5 and SB-2 IL-8-L5, formed detectable tumors earlier and grew in all injected mice (100% tumor uptake, reaching 0.5 to 0.65 cm in mean diameter in 56 ± 5 (SD) days. Subcutaneous tumor tissues from two mice that had been injected with SB-2 IL-8-H5 and SB-2 IL-8-L5 cells were harvested 55 days after injection and assayed for IL-8 expression by Northern blot analysis. This analysis demonstrated that, 55 days after injection, the s.c. tumors of IL-8-transfected cells continued to express IL-8 mRNA *in vivo* (data not shown).

Experimental Lung Metastasis

It has previously been shown that expression of IL-8 directly correlates with metastatic potential in malignant melanoma cells.[21] Here we analyzed the metastatic potential of IL-8-transfected cells in an experimental lung metastasis assay. To that end, 1×10^6 cells were injected into the lateral vein of BALB/c nude mice, and 60

Table 2. Bioactivity of IL-8 Secreted by SB-2 Cells Transfected with the IL-8 Gene

Cell lines	Number of PMNs migrated ± SEM
SB-2 parental	4 ± 1
SB-2 neo	2 ± 1
SB-2 IL-8-H5	233 ± 34 $P<0.001$
SB-2 IL-8-L5	225 ± 30 $P<0.001$
SB-2 IL-8-H10	206 ± 12 $P<0.001$

A total of 1.5×10^5 human PMNs were added to the upper compartment of the Boyden chamber. Supernatants (100 μl) from melanoma cells before and after transfection with the IL-8 gene served as the chemotactic factor in the lower compartment. After 1 hour of incubation, the cells on the lower surface of the filter were stained and counted.

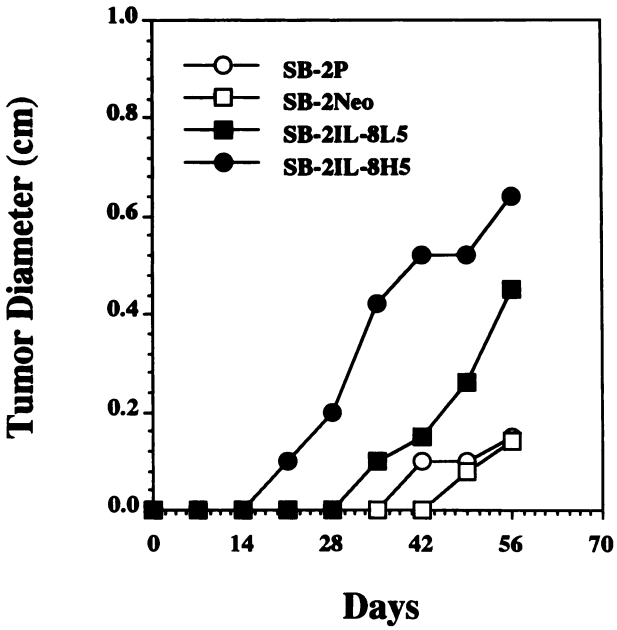

Figure 2. The tumorigenicity of SB-2 parental, neo-transfected cells and two IL-8-transfected clones, 60 days after s.c. injection into nude mice. Tumor cells (1×10^6 in 0.2 ml of HBSS) were injected over the right scapular region. Growth of s.c. tumors was monitored by weekly examination and measurement of tumors with calipers. The data represent the mean diameter observed in five animals per group.

days later, the number of lung metastases was assayed. We found that parental and control neo-transfected cells did not metastasize to the lung (Table 3), whereas SB-2 IL-8-H5 and SB-2 IL-8-L5 cells formed lung metastases in five of five and three of five mice, respectively, given injections. A median of 11 (range, 3 to 24) and 12 (range, 4 to 21) colonies, respectively, were found in the mice.

The increase in tumor growth and ability to produce lung metastases by SB-2 IL-8 cells was not due to differences in cell division time, as no significant differences in cell doubling time were found among the SB-2 parental, SB-2-neo, and IL-8-transfected cell populations cultured *in vitro* in the presence of several different concentrations (0.5, 1, 5, and 10%) of fetal bovine serum (data not shown). These data demonstrate that IL-8 does not act as an autocrine growth factor for the SB-2 cells *in vitro*.

Table 3. Experimental Lung Metastasis of SB-2 Human Melanoma Cells Transfected with IL-8 Gene in Nude Mice

Cell lines	Metastases			*P* value
	Median*	Range	Incidence†	
SB-2 parental	0	0–2	1/5	
SB-2 neo	0	0–1	1/5	
SB-2 IL-8-H5	11	3–24	5/5	<0.001
SB-2 IL8-L5	12	4–21	5/5	<0.001

A total of 2×10^6 cells of each cell line were intravenously injected, and lung metastases were determined 2 months later.

*Median number of lung tumor colonies counted with the aid of a dissecting microscope.

†Number of positive mice per number of injected mice.

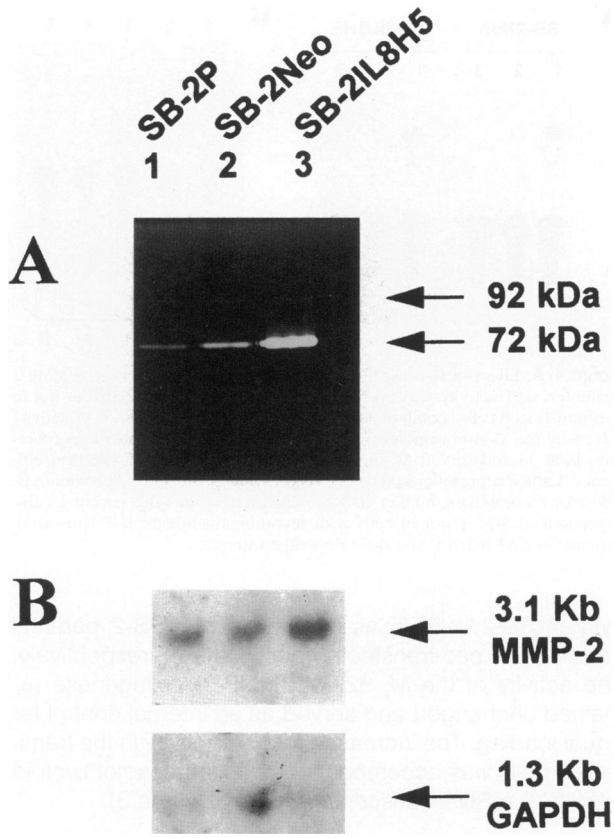

Figure 3. A: Effect of IL-8 gene expression on type IV M_r 72,000 (MMP-2) collagenase activity. IL-8-transfected cells (**lane 3**) displayed a fourfold increase in MMP-2 activity in comparison with SB-2 parental (**lane 1**) and neo-transfected (**lane 2**) cells. The activity of the M_r 92,000 (MMP-2) collagenase remained unchanged and serves as an internal control for equal loading **B:** Northern blot analysis for the expression of MMP-2. MMP-2 mRNA was up-regulated in the IL-8-transfected cells (**lane 3**) in comparison with parental (**lane 1**) and neo-transfected (**lane 2**) cells.

Increase in M_r 72,000 Type IV Collagenase (MMP-2) Activity and mRNA in IL-8-Transfected Cells

We previously demonstrated that UV-B irradiation of SB-2 cells induced IL-8 mRNA production and secretion of IL-8 in these cells.[22] These cells displayed an increase in tumor growth and metastasis in nude mice. Moreover, UV-B irradiation of these cells resulted in increased M_r 72,000 collagenase (MMP-2) activity.[22] As IL-8 has recently been shown to be associated with angiogenesis,[18,19] these results suggested to us that IL-8 may exert its angiogenic activity through the induction of MMP-2 in the tumor cells. To further establish the role of IL-8 in MMP-2 expression and activity, we next analyzed the activity of type IV collagenases (M_r 72,000, or MMP-2, and M_r 92,000, or MMP-9) in SB-2 cells before and after transfection with the IL-8 gene. Supernatants from SB-2 parental, neo-transfected, and SB-2 IL-8-H5 cells were analyzed for collagenase activity by zymography.[27] Collagenase activity in the supernatants was normalized to cell number (see Materials and Methods). The results depicted in Figure 3A show a 4.8- and 3.7-fold increase (determined by densitometry) in MMP-2 collagenase ac-

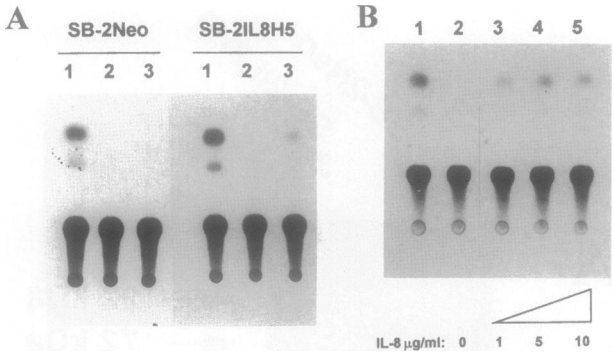

Figure 4. A: Effect of IL-8 expression on CAT activity driven by the MMP-2 promoter. CAT activity was up-regulated in IL-8-transfected cells but not in neo-transfected cells (compare **lane 3** in both cell populations). CAT activity driven by the SV-40 promoter was the same in both cell populations (compare **lane 1**) and served as an internal control for equal transfection efficiency. **Lane 2** represents naked CAT vector without the MMP-2 promoter. **B:** Effect of recombinant human IL-8 on CAT activity in SB-2 parental cells. Treatment of SB-2 parental cells with recombinant human IL-8 caused an increase in CAT activity in a dose-dependent manner.

tivity in SB-2 IL-8-H5 as compared with SB-2 parental (lane 1) and neo-transfected cells (lane 2), respectively. The activity of the M_r 92,000 (MMP-9) collagenase remained unchanged and served as an internal control for equal loading. The increased MMP-2 activity in the transfected cells was accompanied by an increase of twofold in MMP-2 mRNA transcripts (Figure 3B, lane 3).

Effect of IL-8 on the Promoter of MMP-2

To examine the effect of IL-8 on MMP-2 transcription, the MMP-2 promoter (−390 to +291) was linked upstream of the CAT reporter gene and transfected into control, neo-transfected, and IL-8-transfected SB-2 IL-8-H5 cells. Forty-eight hours after transfection, cell extracts were prepared, and equivalent amounts of extracts exhibiting the same β-gal activity were tested for CAT activity. As Figure 4A shows, CAT activity driven by the MMP-2 promoter was detected only in the IL-8-transfected cells and not in neo-transfected cells (compare lane 3 in SB-2 Neo *versus* SB-2IL8H5). CAT activity driven by the SV-40 promoter was the same in both cell populations and served as an additional internal control for transfection efficiency (lane 1 in Figure 4A). We next analyzed whether the increase in CAT activity driven by the MMP-2 promoter was mediated by IL-8. To that end, parental SB-2 cells were transfected with the MMP-2-CAT construct in the presence of different dosages of human recombinant IL-8, and the activity of CAT was determined. The results shown in Figure 4B indicate that IL-8 caused an increase in CAT activity in a dose-dependent manner within a range of 1 to 5 μg/ml and reached a plateau in the presence of 10 μg/ml.

Melanoma Cell Migration through Matrigel-Coated Filters

We next analyzed whether the activation of MMP-2 in the IL-8-transfected cells correlated with an increase in penetration through the basement membrane, an important component in the process of tumor invasion and metastasis. To that end, SB-2 cells before and after transfection with the IL-8 gene were assayed for their potential to penetrate through filters coated with Matrigel. SB-2 parental and neo-transfected cells had a low potential to penetrate through Matrigel-coated filters (Table 4). In contrast, IL-8-transfected cells exhibited increased ability ($P < 0.001$) to invade through these filters. Collectively, these data indicate that activation of type IV collagenase (MMP-2) by IL-8 may provide a mechanism to account for the increase in metastatic potential of SB-2 IL-8-transfected cells.

Table 4. Effect of IL-8 Expression on Migration through Matrigel-Coated Filters

Cell lines	Number of cells migrated ± SEM
SB-2 parental	15 ± 3
SB-2 neo	10 ± 2
SB-2 IL-8-H5	146 ± 41 ($P < 0.001$)
SB-2 IL-8-L5	137 ± 19 ($P < 0.001$)
SB-2 IL-8-L5 (no chemoattractant[b])	9 ± 4

Filters were coated with Matrigel (25 μg/filter). Melanoma cells (2×10^5) were added to the upper compartment of the Boyden chamber. After 6 hours of incubation, the cells on the lower surface of the filter were stained and counted. For SB-2 IL-8-L5, the fibroblast-conditioned medium was replaced with plain medium. *P* values were calculated in comparison with SB-2 parental and neo-transfected cells.

Discussion

We have previously demonstrated a correlation between IL-8 expression and the metastatic potential of human melanoma cells.[21–22] In the present study we show that enforced expression of IL-8 in SB-2 melanoma cells (which express negligible levels of IL-8 mRNA and protein) increased their tumorigenic and metastatic potential in nude mice, thus providing direct evidence for the involvement of IL-8 in metastasis. We used the SB-2 cell line in our studies because it is the only line in our panel that originated from a primary cutaneous melanoma[23] and the only melanoma cell line that produces negligible levels of IL-8 expression. All of the other human melanoma cell lines examined were established from lymph node or brain metastases, expressed IL-8, and had levels of IL-8 mRNA and protein that correlated with their metastatic potential *in vivo*.[21]

The metastatic potential of a tumor depends among many factors on proper vascularization and its ability to degrade type IV collagen. Recently, IL-8 has been shown to be associated with angiogenesis.[18,19] Here we show that IL-8 may exert its angiogenic activity through the induction of type IV collagenase in the tumor cells. Activation of type IV collagenase provides a potential mechanism for the observed increase in the metastatic potential of SB-2 cells after transfection with the IL-8 gene. Indeed, it has recently been shown that MMP-2 (gelatinase A) activity directly modulates melanoma cell adhesion and spreading to extracellular matrix.[33] These results imply that, in addition to contributing to proteolysis of extracellular matrix components, MMP-2 also functions

to proteolyze cell surface components that mediate attachment of melanoma cells to the extracellular matrix. As such, MMP-2 may modulate cell adhesion and facilitate cell migration and invasion.[33]

Several possible mechanisms for the up-regulation of MMP-2 expression by IL-8 should be considered. IL-8 has recently been shown to be capable of activating phospholipase D in human neutrophils.[34] Phospholipase D, in turn, is involved in the intracellular signal transduction pathway that mediates induction of MMP-2 by laminin in tumor cells.[35] IL-8, therefore, can increase type IV collagenase activity, thus contributing to host invasion and metastasis. Indeed, our data demonstrate an increase in MMP-2 mRNA and activity in SB-2 cells transfected with their IL-8 gene. IL-8 is likely to affect MMP-2 expression at the transcriptional level as CAT activity driven by the MMP-2 promoter was up-regulated in SB-2 IL-8-transfected cells and in SB-2 parental cells treated with human recombinant IL-8 (Figure 4). As IL-8 expression in melanoma directly correlates with metastatic potential, these observations support previous findings about linkage between type IV collagenase and the malignant phenotype.[36–38] The MMP-2 promoter contains two Sp1-binding sequences and one Ap-2-binding motif.[28] IL-8 could alternatively influence the transcription of MMP-2 by regulating the expression of the transcription factors that bind to these sites. In addition to the activation of type IV collagenase by IL-8, we cannot exclude the possibility that IL-8 can affect angiogenesis by other mechanisms, however; for example, it has recently been shown that IL-8 specifically binds to human endothelial cells but not to smooth muscle cells,[39] suggesting a direct role in proliferation and function of endothelial cells. In addition, Schadendorf et al[40] have recently demonstrated that IL-8 produced by human melanoma cells is an essential autocrine growth factor. In our studies, however, transfection of the SB-2 cells with the IL-8 gene did not change their proliferation rate *in vitro*, suggesting that IL-8 does not act as an autocrine growth factor for the SB-2 cells.

Our previous observation[21,22] and the present study point to a close association between IL-8 secretion and the metastatic potential of intravenously injected human melanoma cells into nude mice. These data seem to be at first glance in contrast to a recent report by Schadendorf and his colleagues who found no correlation between metastatic capacity and IL-8 secretion.[41] This might be true for the production of spontaneous metastases after s.c. injections whereas the ability of melanoma cells to produce either lymph node, liver, or lung metastases after intravenous injections was correlated with the levels of IL-8 production.[41] Our observation is further supported by the recent finding that the levels of serum IL-8 was found to be elevated in patients with metastatic melanoma and to be correlated with tumor load.[42]

Our present data indicate that the role of IL-8 in the progression of melanoma is more complex than that of a growth factor. The observation that diverse inflammatory signals, including IL-1 and tumor necrosis factor, which are produced by keratinocytes,[43,44] can induce IL-8 production in tumor cells[45] supports the hypothesis that angiogenesis in the area surrounding s.c. melanoma lesions could be due to overexpression of IL-8. The finding that interferon-β prevents the up-regulation of IL-8 expression induced by IL-1 and tumor necrosis factor-α[46] points to the usefulness of interferon-β as an antiangiogenic agent that may prevent tumor growth and metastasis of melanoma cells.

Malignant tumors do not grow beyond 2 to 3 mm^3 and cannot metastasize unless there is stimulation for the formation of new blood vessels.[47] Angiogenic factors produced by tumor or host cells are critical to the formation of a vascular bed necessary to support tumor growth at the primary or metastatic sites. The two most potent angiogenic molecules are vascular endothelial growth factor (VEGF) and bFGF. We studied the expression of VEGF and bFGF in human melanoma cell lines with different metastatic capabilities. Only low levels of these angiogenic molecules were found, and no discernible differences in expression of these factors were found among the lines tested.[14] Moreover, in the present study, we did not observe any changes in the levels of mRNA for VEGF and bFGF after transfection with the IL-8 gene (data not shown), indicating that these factors did not play a role in the increased metastatic potential of SB-2 cells after IL-8 expression.

The development of malignant melanoma in human beings progresses through a multistage process (reviewed in Ref. 48). During recent years, the discrete steps of this progression have been better defined at the molecular level. Among the responsible molecules are the major histocompatibility antigens,[49] the intracellular adhesion molecules ICAM-1 and MCAM (MUC18),[31,50,51] the integrin receptor family,[52,53] and the tyrosine kinase receptor c-KIT,[54–57] and several cytokines such as IL-6[12,58] and IL-10[14] have the potential to control the growth of melanoma cells. The roles of the two tumor suppressor genes *p53* and *p16* (CDKN2), however, are not yet clear.[32,59] The present study and previous studies from our group[21,22] suggest that in this tumor system IL-8 may serve as the angiogenic factor distinguishing benign from malignant cells.

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